# ALTERATIONS IN CARDIOMYOCYTE FUNCTION AFTER PULMONARY TREATMENT WITH STAINLESS STEEL WELDING FUME IN RATS

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## Abstract

Welding fume is composed of a complex of different metal particulates. Pulmonary exposure to different welding fumes may exert a negative impact on cardiac function, although the underlying mechanisms remain unclear. To explore the effect of welding fumes on cardiac function, Sprague-Dawley rats were exposed by intratracheal instillation to 2 mg/rat of manual metal arc hard surfacing welding fume (MMA-HS) once per week for 7 wk. Control rats received saline. Cardiomyocytes were isolated enzymatically at d 1 and 7 postexposure. Intracellular calcium ($[Ca^{2+}]i$) transients (fluorescence ratio) were measured on the stage of an inverted phase-contrast microscope using a myocyte calcium imaging/cell length system. Phosphorylation levels of cardiac troponin I (cTnI) were determined by Western blot. The levels of nonspecific inflammatory marker C-reactive protein (CRP) and proinflammatory cytokine interleukin-6 (IL-6) in serum were measured by enzyme-linked immunosorbent assay (ELISA). Contraction of isolated cardiomyocytes was significantly reduced at d 1 and d 7 postexposure. Intracellular calcium levels were decreased in response to extracellular calcium stimulation at d 7 postexposure. Changes of intracellular calcium levels after isoprenaline hydrochloride (ISO) stimulation were not markedly different between groups at either time point. Phosphorylation levels of cTnI in the left ventricle were significantly lower at d 1 post-exposure. The serum levels of CRP were not markedly different between groups at either time point. Serum levels of IL-6 were not detectable in both groups. Cardiomyocyte alterations observed after welding fume treatment were mainly due to alterations in intracellular calcium handling and phosphorylation levels of cTnI.

Welding is a common industrial process that is used to join metals at extremely high temperatures. Importantly, the process generates potentially hazardous metal fumes and gases (Gordon, 2004; Antonini et al., 2004). Approximately 340,000 workers were classified

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as full-time welders in the United States in 2010 (Bureau of Labor Statistics, 2013). Millions of workers worldwide perform duties related to welding operations but are not classified as full-time welders. The number of full-time welders in the United States is expected to grow to more than 400,000 workers by the year 2020.

Welding fume generation involves the vaporization of the metals and oxides of an electrode or wire that is consumed during the process. Rapid condensation of the vapors follows, generating particulates composed of different metal oxides that depend on the composition of the electrode (Harris, 2002). The process by which high-temperature metal vapors are transformed into primary particles is called nucleation (Zimmer, 2002). Nucleation is followed by coagulation where smaller primary particles collide to form larger chain-like structures. The primary particles formed during welding are in the ultrafine size range ($<0.1$ μm) (Sowards et al., 2010). Three modes of particle sizes have been described during welding: (1) a nucleation nanometer-sized mode ($\sim0.01$–$0.1$ μm) of individual primary particles, (2) an accumulation mode ($0.10$–$1$ μm) of agglomerated and coalesced particles formed from the nucleation mode, and (3) a coarse mode ($\sim1$–$20$ μm) of nonagglomerated, more spherical particles (Zimmer and Biswas, 2001).

A link exists between environmental pulmonary exposure to particulate matter (PM) air pollution and adverse cardiovascular outcomes in epidemiological studies (Robert at al., 2010; Liao et al., 2011; Beckerman et al., 2012). When levels of PM are elevated, hospitalizations for cardiovascular events were found to increase (Chang et al., 2013; Chiu et al., 2013; Yang, 2008). Because welding processes generate inhalable metal fumes, welders are at risk for the development for adverse cardiovascular effects. Until recently, few reports on the study of cardiovascular health in welders existed (Sjögren et al., 2002; Ibfelt at al., 2010; Scharrer et al., 2007; Cavallari et al., 2008; Fang et al., 2009; Erdely et al., 2011; Antonini, 2014). Most of these studies indicated that exposure to welding particles led to cardiovascular alterations. However, little mechanistic information is available in regard to the cardiovascular responses associated with welding fume exposure. Thus, additional research is needed.

The goal of the current study was to examine whether there is a difference in cardiomyocyte function after pulmonary exposure to welding fume in an animal model. It was postulated that solubilized, bioavailable metals or associated bioactive cofactors translocate from lungs after welding fume deposition, enter the circulation, and are transported to the heart. At different time points after exposure, parameters of cardiomyocyte function were examined, including expression of phosphorylated cardiac troponin I and different inflammatory mediators such as serum C-reactive protein (CRP) and interleukin 6 (IL-6). The effects of fractional shortening (FS) and change in calcium transients when treated with extracellular $Ca^{2+}$ and isoprenaline (ISO) also were measured. Troponin, calcium handling, and fractional shortening are important factors in the regulation of cardiac contractility. This study is unique in that it is the first to examine the effects of pulmonary welding fume treatment on specific endpoints of cardiomyocyte function.

## METHODS

### Welding Fume Collection and Characterization

A bulk sample of a specific welding fume was collected by Lincoln Electric Co. (Cleveland, OH). The fume was generated in a cubical open front fume chamber (volume = 1 $m^3$) by a skilled welder performing manual metal arc welding using a flux-covered stainless steel hard surfacing electrode (MMA-HS; Wearshield 15CrMn, Lincoln Electric Co., Cleveland, OH) and collected on 0.2-μm Nuclepore filters (Nuclepore Co., Pleasanton, CA). Particle size of the welding sample was characterized by scanning electron microscopy and was of respirable size with count mean diameters of <1 μm. After collection, a small portion of the MMA-HS fume sample was digested, and metals were analyzed by inductively coupled plasma–atomic emission spectroscopy (ICP-AES), according to National Institute for Occupational Safety and Health (NIOSH) method 7300 (NIOSH, 1994). The percentages by weight of total metals present in the MMA-HS welding fume used in the current study were 19.3% Fe, 50.9% Mn, 8.46% Cr, 12.1% K, 6.73% Na, and 0.09% Ni, as determined previously (Antonini et al., 2010).

In addition, the MMA-HS fume sample was suspended in distilled water, pH 7.4, and sonicated for 1 min with a Sonifier 450 cell disruptor (Branson Ultrasonics, Danbury, CT). The particle suspension (total sample) was incubated for 24 h at 37°C, and the sample was centrifuged at 12,000 g for 30 min. The supernatant of the sample (soluble fraction) was recovered and filtered with a 0.22-μm filter (Millipore Corp., Bedford, MA). The pellet (insoluble fraction) was resuspended in water. The sample suspensions (total, soluble, and insoluble fractions) were digested, and metals were analyzed by ICP-AES by the Division of Applied Research and Technology (DART, Cincinnati, OH) according to NIOSH method 7300 (NIOSH, 1994). The soluble/insoluble ratio for the sample was 0.218. The soluble fraction was composed of primarily 56% K, 25.4% Na, and 15.1% Cr.

### Animals

Male Sprague-Dawley [Hla:(SD) CVF] rats from Hilltop Lab Animals (Scottdale, PA), weighing 250–300 g and free of viral pathogens, parasites, mycoplasmas, *Helicobacter*, and CAR Bacillus, were used for all exposures. The rats were acclimated for at least 6 d after arrival, housed in ventilated polycarbonate cages on Diamond-Dri cellulose chips and hardwood Sani-chips as bedding, and provided HEPA-filtered air, irradiated Teklad 2918 diet, and tap water ad libitum when not being exposed. Our animal facilities are specific pathogen free, environmentally controlled, and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All animal procedures used during the study were reviewed and approved by the institution's Animal Care and Use Committee.

### Animal Treatment

Rats were anesthetized by an intraperitoneal injection of 0.6 ml of a 1% solution of sodium methohexital (Brevital, Eli Lilly, Indianapolis, IN) and intratracheally instilled once per week for 7 wk with 2 mg/rat of the MMA-HS welding fume in 300 μl sterile phosphate-buffered saline (PBS). Vehicle control animals were intratracheally instilled with 300 μl

sterile PBS. Intratracheal instillation dose of 2 mg/rat per week was selected based upon results from a previous dose-response welding fume study (Antonini et al., 1996).

Assuming fume concentration (5 mg/m$^3$, previous threshold limit value for welding fume), human minute ventilation volume (20,000 ml/min $\times$ 10$^{-6}$ m$^3$/ml), exposure duration (8 h/d $\times$ 60 min/h), and deposition efficiency (15%), it was calculated that the daily lung burden of a welder is approximately 7.2 mg. Using the surface area of alveolar epithelium (rat = 0.4 m$^2$; human = 102 m$^2$; Stone et al., 1992) as a dose metric, the daily lung burden for a similar exposure in the rat is 0.0282 mg; 2 mg/0.0282 mg = 71 d of a worker exposed at 5 mg/m$^3$ for 8 h/d.

### Cardiomyocyte Collection and Analysis

Cardiomyocyte isolation and measurements were conducted at different time points (1 or 7 d after treatment). Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (26 mg/kg body weight [bw]; Fort Dodge Animal Health, Fort Dodge, IA). Hearts were quickly removed and perfused with Krebs–Hensleit bicarbonate (KHB), containing (in m$M$) 118.1 NaCl, 3KCl, 1.2 CaCl$_2$, 1.2 MgSO$_4$, 1 KH$_2$PO$_4$, 27.3 NaHCO$_3$, 10 glucose, and 2.5 pyruvic acid, pH 7.4, according to the Langendorff method at a constant rate of 5–8 ml/min for 15 min using a peristaltic pump. Then hearts were perfused with low-calcium KHB containing (in m$M$) 105.1 NaCl, 3 KCl, 0.01 CaCl$_2$, 1.2 MgSO$_4$, 1 KH$_2$PO$_4$, 20 NaHCO$_3$, 10 glucose, 5 pyruvic acid, 10 taurine, and 5 mannitol, pH 7.3, for another 15 min to dilate the vessels. The hearts were immersed in recirculating low-calcium KHB containing collagenase (0.08 mg/ml; Boehringer Mannheim Biochemicals, Indianapolis, IN) for 40–50 min to be digested. All of the buffer and enzyme solutions were maintained at 39°C and pre-equilibrated with 95% O$_2$–5% CO$_2$. When the hearts became soft, the ventricles were cut and placed with the enzyme solution. The ventricles were cut such that the tissue was spread out, but still intact. The tissue was then placed into low-calcium KHB and agitated until it fell apart. After the cells had settled for 10 min, the supernatant was removed and replaced with increasing amounts of KHB solution. Then, 10% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) was gradually added to replace the KHB solution to help repair the cell membranes. This procedure was continued until 80% of the preparation consisted of viable cells. Finally, tissue mixture was passed through a 225-μm nylon mesh. Cells were viewed by light microscopy, and only cells shaped like striated rods with no contractions were selected for the study.

β-Adrenergic stimulation produces enhanced systolic and diastolic function in cardiac myocytes. The cardiomyocytes were perfused with increasing concentrations of ISO, 10$^{-10}$ to 10$^{-6}$ $M$ (Sigma-Aldrich, St. Louis, MO). Poor calcium handling, including defective release and storage of Ca$^{2+}$ in the sarcoplasmic reticulum, contributes to cardiac dysfunction. To test the effect of welding fume exposure on calcium handling, increasing concentrations of extracellular Ca$^{2+}$ (1.2, 2.4, 3.6 m$M$) were used to perfuse the cardiomyocytes. Myocytes from the exposed and control groups were compared to see if there was a difference in the percent FS and percent change in calcium transients when extracellular Ca$^{2+}$ concentrations were increased. Data were compared using analysis of

variance followed by pairwise comparisons between control and treated groups using Student's *t*-test. Differences were considered statistically significant at the level of $p < .05$.

### Cardiac Tissue Collection and Serum Analysis

At d 1 and 7 after the 7 wk of exposure, cardiac tissue and serum from treated animals were collected. The CRP and IL-6 in serum were determined using the following methods: ELISA CRP Kit (eBioscience catalog number 88–7501) and ELISA IL-6 Kit (Invitrogen Catalog number KRC0061). The changes in concentration of phosphorylated cardiac troponin I as a biomarker in cardiac tissue were examined by Western blot.

Primary antibody antirabbit phosphorylated cardiac troponin I (1:2000) cell signaling was used and incubated overnight on the shaker at 4°C, and secondary anti-rabbit ummunoglobulin G (IgG) peroxidase-linked species-specific F(ab′)2 antibody(1:5000) was added for 1.5 h on the shaker. ECL Kit Amersham ECL Prime Western Blotting Detection reagent RPN 2232 was prepared by adding 1 ml solution A and 1 ml solution B in a small box. Different exposure times were examined, starting with 15 s, increasing time to 2 min or more, adjusting the exposure time according to band shade. After obtaining x-ray film (shown later in Figures 4 and 5 for the left and right ventricle) of the polyvinylidene fluoride (PVDF) membrane, films were scanned on the computer with an Image J program. The mean of the phosphorylated cardiac troponin I/β actin ratio, standard deviation, and standard error were computed for the group of rats exposed to welding fumes and control.

The Wilcoxon statistical test was used to compare the ratio of phosphorylated cardiac troponin I and β-actin between the exposed and control groups of rats, as well as the levels of CRP and IL-6 between the exposed and control groups of rats. Differences were considered statistically significant at the level of $p < .05$.

## RESULTS

For Figures 1 and 2, $[Ca^{2+}]$ concentrations were not measured. The software that was used measured the ratio of fluorescence when $[Ca^{2+}]$ binds to the dye, thus estimating the amount of intracellular $[Ca^{2+}]$. The percent change from basal $[Ca^{2+}]i$ ratio is for fluorescence ratios. Figures 3 and 4 show the concentrations of extracellular $[Ca^{2+}]$ was exposed to the cells.

Figures 1 and 2 show the concentration-response curve of percent change from baseline fractional shortening (FS) and $[Ca^{2+}]i$ in response to ISO at d 1 and 7 postexposure, respectively. The results show that the concentration-response curve for % change from baseline (FS) was significantly different at d 1 and 7 when comparing the welding fume group with control (Figures 1A and 2A). However, $[Ca^{2+}]i$ in response to ISO was not markedly different when comparing the two groups at d 1 and 7 postexposure (Figures 1B and 2B).

Figures 3 and 4 show bar graphs depicting percent change from baseline FS and $[Ca^{2+}]i$ change in response to increased $Ca^{2+}$ concentration at d 1 and 7 postexposure, respectively. The percent change from baseline FS was significantly reduced in response to elevated

Ca$^{2+}$concentration of 2.4 and 3.2 m*M* at 1 and 7 d after welding fume exposure compared to control (Figures 3A and 4A). In addition, the [Ca$^{2+}$]i change in response to increased Ca$^{2+}$concentration of 2.4 mM was significantly decreased at d 7 post exposure to welding fume (Figure 4B).

The results for phosphorylated cardiac troponin I to β-actin ratio for left and right ventricle for control versus welding fume at 1 and 7 d postexposure are shown in Figures 5 and 6, respectively. As noted in Figure 5, phosphorylated cardiac troponin I and β-actin ratio was significantly lower in the exposed compared to control group for the left ventricle at d 1 postexposure. No marked difference was observed in the phosphorylated cardiac troponin I and β-actin ratio in the right ventricle comparing the two groups (Figure 6).

The serum levels of CRP for the control and welding fume groups at 1 and at 7 d post-exposure are shown in Figure 7. CRP levels in serum for the two groups were not markedly different. Serum levels of IL-6 were not detectable in both the welding-fume-treated and control groups (data are not shown).

## DISCUSSION

Hospitalizations for adverse cardiovascular episodes increase when ambient levels of PM are elevated (Hsieh et al., 2010; Robert at al., 2010). Welding represents a unique occupational PM exposure because of the generation of inhalable metal fumes. When inhaled in the lungs, solubilized metals and/or bioactive and inflammatory cofactors induced by the pulmonary deposition of metal-rich welding particles may reach the circulatory system and enter vital organs (e.g., heart), possibly producing functional alterations or damage. Moreover, soluble bioavailable components of residual oil fly ash, a metal-rich PM, were found to alter cardiomyocyte growth, function, and viability, as well as inducing oxidative stress (Knuckles and Dreher, 2007). The MMA-HS fume used in the study was highly water-soluble and composed of primarily Fe, Mn, and Cr (8.46%). A significant amount of soluble Cr and alkali metals (K, Na) present in the flux material was also present in this particular welding fume. Because the MMA-HS fume is highly water-soluble, it is conceivable that the metals associated with this fume might be readily bioavailable and likely distribute freely to other organ systems. Antonini et al. (2010) showed that various metals of the MMA-HS fume were cleared from the lungs at different rates, suggestive of material separation after particle deposition in the lungs. The deposited metals were cleared quickly from the lungs after treatment with the fume. There were significant elevations of Mn and Cr in the blood and lung-draining lymph nodes at 1 d after MMA-HS fume exposure. Fe was significantly elevated in the lung-draining lymph nodes at 1 d after the last treatment, remaining elevated up to 35 d. The metals were observed to translocate from the lungs to other organs. Mn was significantly elevated in the heart, kidneys, and spleen at 1 d after the last treatment. Cr was significantly elevated in kidneys and spleen 1 d after exposure and remained elevated in the spleen throughout the 105-d recovery period after treatment.

Epidemiological studies indicated that exposure to welding fume particles may pose a risk for development of cardiovascular disease. Using two large cohorts from the Swedish

National Censuses of 1970 and 1990, Sjorgen and colleagues (2002) observed a significant increase in mortality rate among welders due to ischemic heart disease. Similarly, Ibfelt at al. (2010) sampled more than 10,000 metal workers in 75 welding companies in Denmark in a prospective cohort and noted a significant rise in hazard rate ratio for chronic ischemic heart disease in welders with increasing exposure to metal particles Studies of welders suggested potential mechanisms related to cardiovascular disease, including effects on heart-rate variability, aortic augmentation index (a marker of arterial stiffness), and markers of systemic inflammation and oxidative stress (Scharrer et al., 2007: Cavallari et al., 2008; Fang et al., 2009).

In the current study, the effects of welding fume exposure on myocyte contractility, $Ca^{2+}$ handling, injury, and inflammation were evaluated. Myocyte injury may result from severe ischemia, and may also be a consequence of oxidative stresses on the myocardium (Mehra et al., 2005). Inflammation is important in the pathogenesis and progression of many forms of heart failure. Therefore, a significant amount of research related to biomarkers of inflammation has been performed (Mehra et al., 2005). Increased serum levels of proinflammatory cytokines, such as IL-6, depress cardiac contraction and induce left ventricular dysfunction. In addition, serum CRP is a nonspecific marker for inflammation and tissue damage and was related to a variety of cardiovascular pathologies, such as congestive heart failure. Moreover, systemic inflammation as measured by serum CRP was found to exert a possible modulatory effect on exposure and heart rate variability in a cohort of welders (Fang et al., 2009).

Animal studies indicate that components common in welding fume may exert cardiovascular effects. Compounds of barium are used as flux components for certain metal arc-welding processes. Hicks et al. (1986) demonstrated that exposure to solutions of barium salts in anesthetized guinea pigs produced marked elevation in resistance to ventilatory airflow, and electrocardiograph (ECG) abnormalities indicating myocardial hyperexcitability. Nickel, a metal commonly found in welding factories, has potential cardiotoxicity, while all-*trans*-retinoid acid promote myocardial recovery (Lou et al., 2013). Subchronic manganese(II) chloride ($MnCl_2$) exposure induced chicken heart damage as a result of mitochondrial metabolism disruption and alterations in ion homeostasis (Shao et al., 2012). Other animal studies indicated that Mn produced acute cardiodepression and hypotension as a result of mitochondrial damage and interaction with $Ca^{2+}$ channels in the cardiovascular system (Yueming and Wei, 2005). Importantly, it also was observed that exposure to stainless steel welding fumes enhanced the development of atherosclerotic lesions in apolipoprotein E knockout (apoE(−/−)) mice. These effects were accompanied by lung inflammation and indications of systemic inflammation and oxidative stress (Erdely et al., 2011).

In the current study, it was demonstrated that pulmonary exposure to welding fume decreased contractility of isolated cardiomyocytes, suggesting a significant effect of welding fume on the heart. Intracellular $Ca^{2+}$ concentrations and phosphorylation levels of cardiac Troponin I are important in regulation of cardiac muscle contraction. Alterations in $Ca^{2+}$ handling and decreased cardiac troponin I phosphorylation result in a reduction in cardiac contractility. In addition, intracellular $Ca^{2+}$ levels were significantly lowered in response to elevated extracellular $Ca^{2+}$ at 7 d post exposure to welding fume, and level of cardiac

troponin I phosphorylation was reduced in the left ventricle at 1 d post exposure to welding fume. These results suggest that decreased myocyte contractility might be due to diminished phosphorylation levels of cTnI at 1 d postexposure and impaired $Ca^{2+}$ handling at 7 d postexposure.

Our data also indicated that serum levels of either CRP nor IL-6 were not affected in rats exposed to welding fume. These findings do not provide evidence for overt inflammation after pulmonary welding fume exposure in our animal model but do not exclude the possibility of effects from other mediators such as oxidized lipids. The lack of pronounced systemic inflammation is in agreement with the findings of Scharrer et al. (2007), who found that blood leukocyte numbers, cell differentials, and blood levels of fibrinogen, CRP, antithrombin III, factor VIII, von Willebrand factor, ristocetin cofactor, sICAM-1, tumor necrosis factor alpha, IL-6, IL-8, and epithelial neutrophil activating peptide 78 were not markedly altered by welding fume inhalation (Scharrer et al., 2007). However, in a study of acute systemic inflammatory responses to welding fume exposure, Kim et al. (2005) measured CRP, fibrinogen, and white blood cell (WBC) levels in blood samples collected at baseline and after 5.3 h of exposure in 24 welders and 13 nonexposed controls. Smokers made up 42% of the welders and 23% of the nonexposed controls. In both smokers and nonsmokers, CRP levels were significantly increased 16 h after welding exposure. The results showed that PM concentration was significantly associated with absolute neutrophil counts in nonsmokers, as well as with CRP levels in both nonsmokers and smokers (Kim et al., 2005).

## Summary

Intracellular $Ca^{2+}$ levels were reduced in response to extracellular $Ca^{2+}$ stimulation at 7 d after pulmonary exposure to welding fume. Pulmonary exposure to MMA-HS welding fume also decreased phosphorylation levels of cardiac troponin I in the left ventricle at 1 d postexposure. The serum levels of CRP were not markedly changed at 1 and 7 d post exposure to welding fume compared with control. Serum levels of IL-6 were not detectable in both welding fume-treated and control groups (data not shown). Welding fume exposure-induced alterations in cardiomyocyte function in this animal model were mainly due to alterations in intracellular $Ca^{2+}$ handling and phosphorylation levels of cardiac troponin I.

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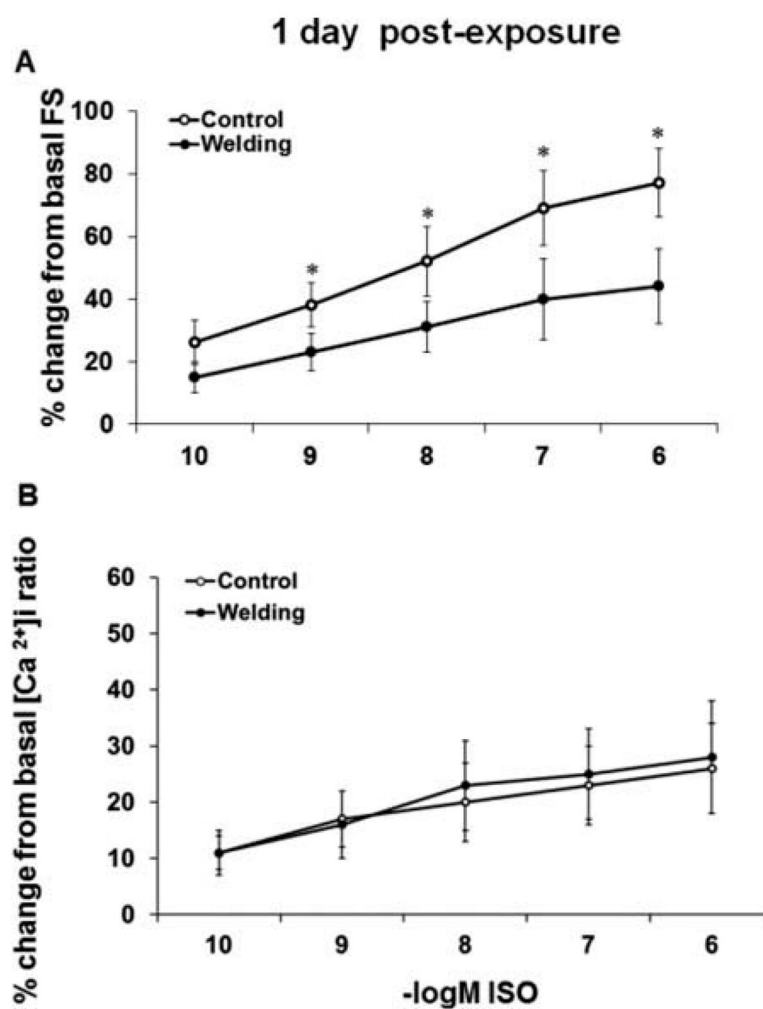
**FIGURE 1.**

The concentration-response curve of percent change from baseline FS (A) and $[Ca^{2+}]i$ (B) in response to ISO at 1 d postexposure. Each value represents the mean $\pm$ SD of 18–24 cardiomyocytes isolated from 4 rats; asterisk indicates significant difference at $p < .05$ compared with control group.
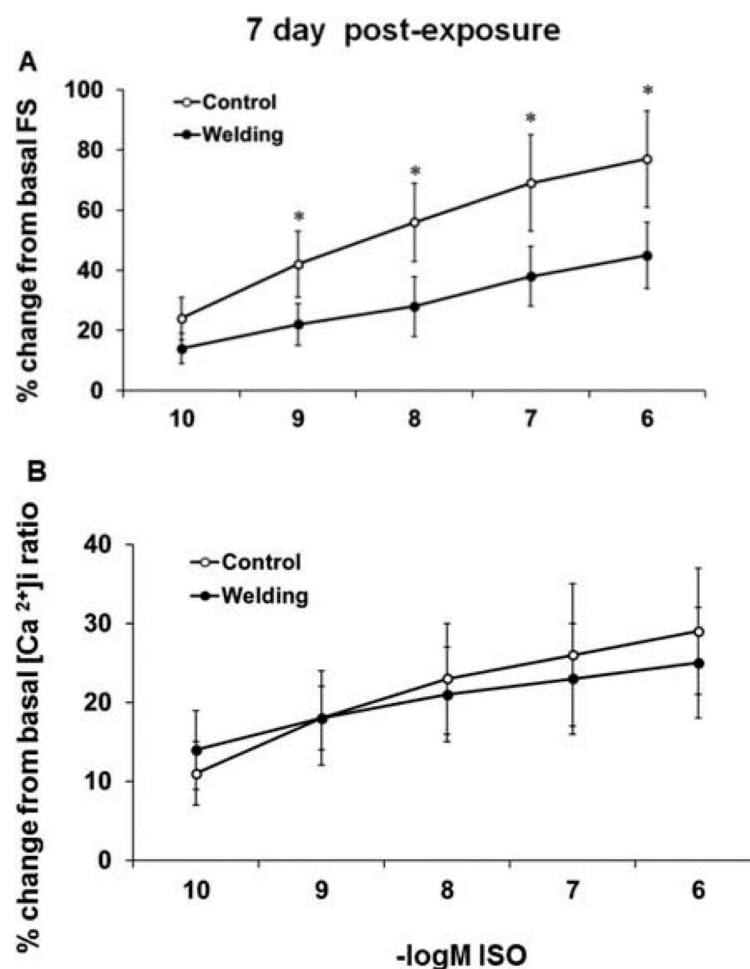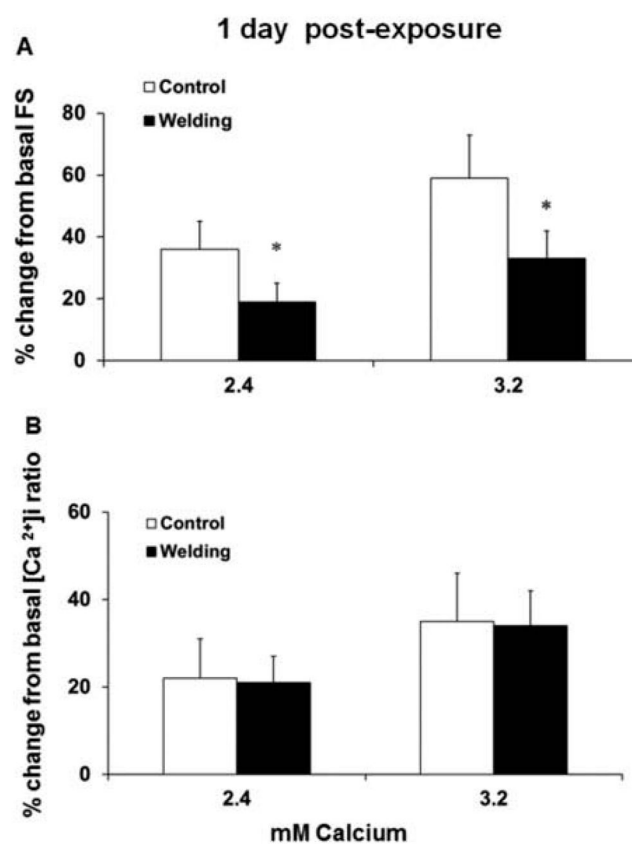
**FIGURE 2.**
The concentration-response curve of percent change from baseline FS (A) and $[Ca^{2+}]i$ (B) in response to ISO at 7 d postexposure. Each value represents the mean ± SD of 18–24 cardiomyocytes isolated from 4 rats. asterisk indicates significant difference at $p < .05$ compared with control group.
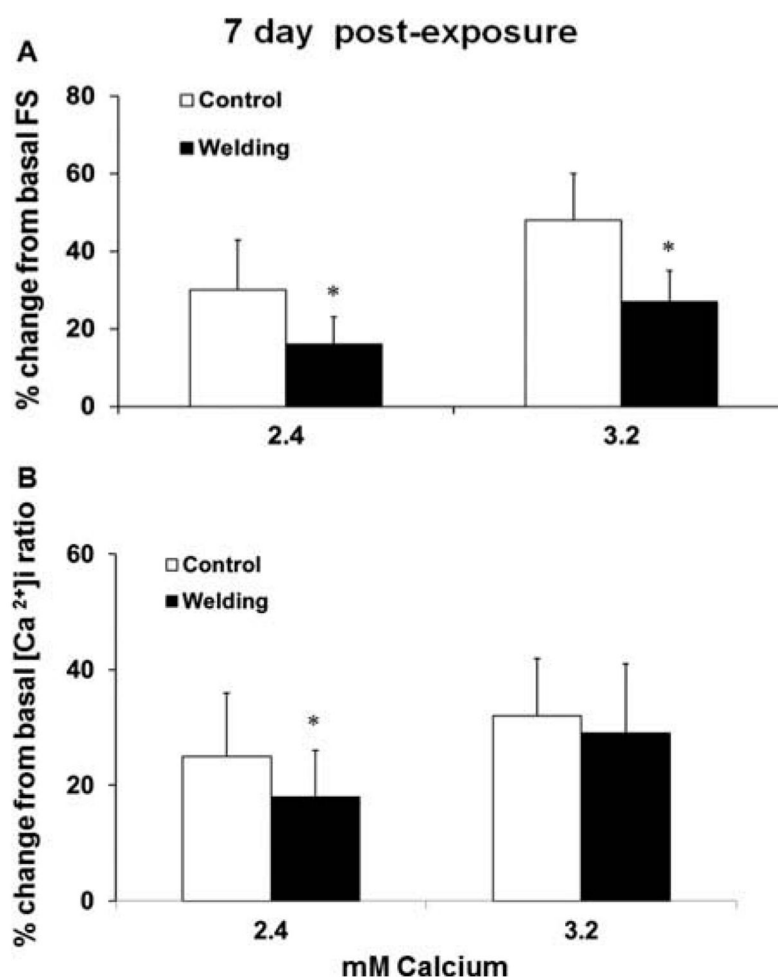
**FIGURE 3.**

Bar graph depicting percent change from baseline FS (A) and $[Ca^{2+}]i$ change (B) in response to increased calcium concentration at 1 d postexposure. Each value represents the mean ± SD of 18–24 cardiomyocytes isolated from 4 rats. asterisk indicates significant difference at $p < .05$ compared with control group.
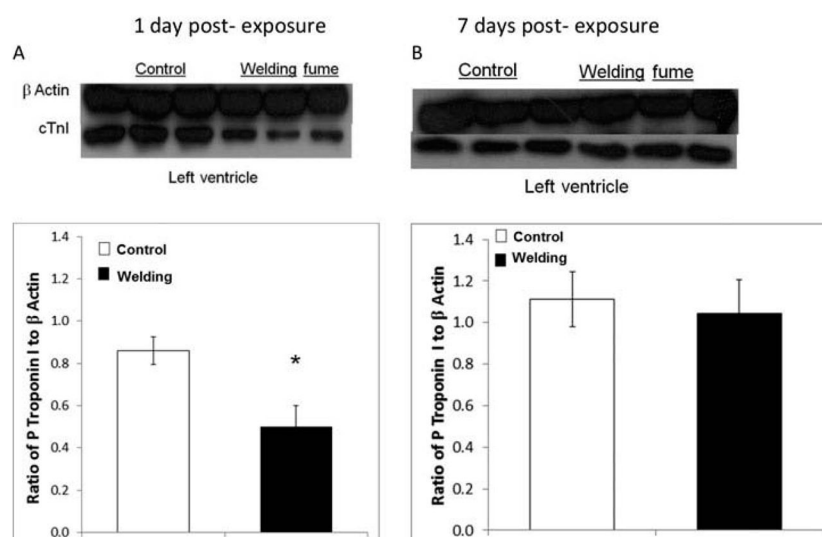
**FIGURE 4.**
Bar graph depicting % change from baseline FS (A) and $[Ca^{2+}]i$ change (B) in response to increased calcium concentration at 7 d postexposure. Each value represents the mean ± SD of 18–24 cardiomyocytes isolated from 4 rats. asterisk indicates significant difference at *p* < .05 compared with control group.

**FIGURE 5.**
Phosphorylated cardiac troponin I to β-actin ratio for left ventricle for control vs. welding fume at (A) 1 d postexposure and (B) at 7 d postexposure (asterisk indicates significantly different from control, $p < .05$; $n = 6$ rats).
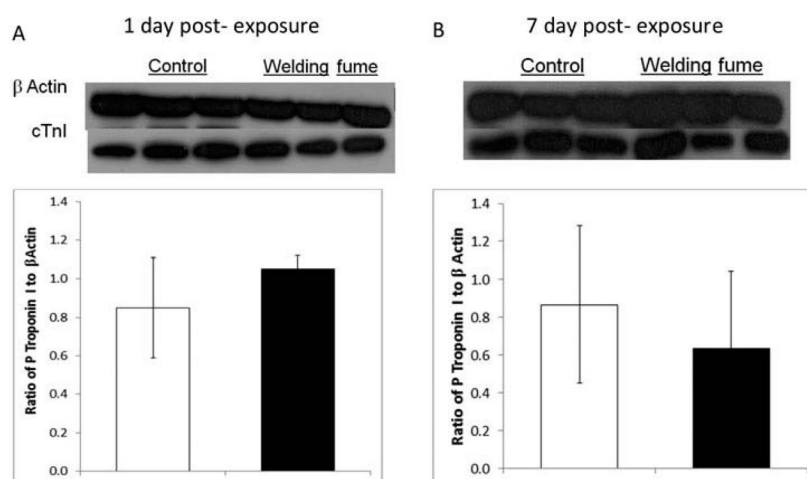
**FIGURE 6.**
Phosphorylated cardiac troponin I to β-actin ratio for right ventricle for control vs. welding fume at (A) 1 d postexposure and at (B) 7 d postexposure ($n = 6$ rats).
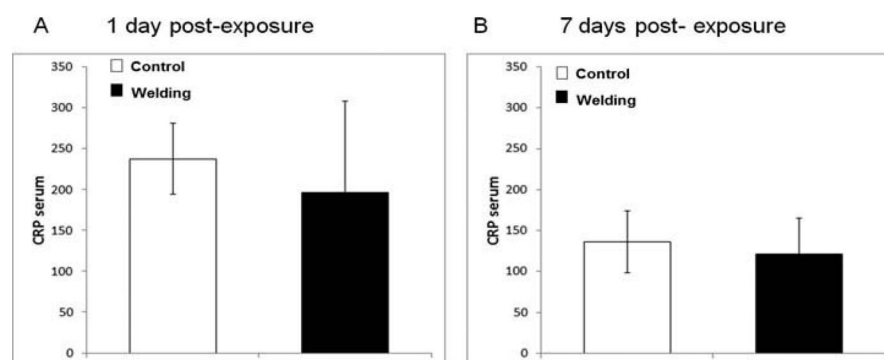
**FIGURE 7.**
CRP serum level for control vs. welding fume (A) at 1 d postexposure and (B) at 7 d postexposure ($n = 12$ rats).